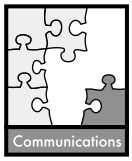

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Taking the stress out of blood collection: comparison of field blood-sampling techniques for analysis of baseline corticosterone

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Many ecological studies use stress hormones to assess the condition, health or disturbance levels of wild organisms. Common blood sampling protocols for this research involve trapping individuals and taking blood within three minutes to obtain a "baseline" for analysis of stress hormones ("conventional method"). In some situations it may be difficult to get an accurate measure of baseline values; therefore, alternative sampling techniques may be preferable. We compared corticosterone levels in samples taken via a newly developed, minimally invasive blood sampling technique with corticosterone levels in blood taken via the conventional method. We collected samples from incubating adult common terns *Sterna hirundo* via blood sucking bugs (Heteroptera, Triatominae) contained in "dummy eggs" ("bug method") and compared measured corticosterone concentrations to concentrations in blood taken from the same birds using the conventional method. We found no significant differences in mean or variance of baseline corticosterone levels between samples collected via the different methods. This suggests that the bug method offers a viable alternative for hormone sampling.

Measuring stress hormone levels in wild organisms is becoming an important tool for ecological studies. Corticosterone levels in blood plasma have been shown to be an index of stress in birds and have been used to determine reactions to trapping, migratory readiness, and the effects of environmental perturbations or human disturbance (Hofer and East 1998, Lohmus et al. 2003, Walker et al. 2005). Use of this tool, however, requires accurate measurement of baseline corticosterone levels for unstressed animals which sometimes can be difficult to obtain (Romero and Reed 2005).

The conventional method for obtaining blood samples to determine baseline corticosterone is to trap an individual and draw blood within three minutes ("conventional method", e.g. Wingfield and Romero 2001). However, in some cases stress from investigator disturbance, including unsuccessful trapping efforts, can make it difficult to obtain accurate measures of baseline corticosterone (Millspaugh and Washburn 2004, Romero and Reed 2005), and may lead to nest desertion (Nisbet 1981, Arnold 1998). Logistical difficulties (e.g. small blood vessels) may also prevent investigators from obtaining a sample within three min. of trapping (Voigt et al. 2004).

Fecal samples offer a non-invasive alternative to investigate hormone levels (Palme 2005). However, as levels of fecal hormone metabolites reflect the release and elimination of hormones over several hours, the time specificity associated with blood sampling techniques may be preferred (Romero and Reed 2005, Touma and Palme 2005). Fecal methods may also have logistical limitations as it may be difficult to link the sample to an individual or to identify the age of the sample (Millspaugh and Washburn 2004).

In this study, we collected blood from incubating common terns *Sterna hirundo* by placing dummy eggs containing blood sucking Triatomine bugs *Dipetalogaster maximus* in nests ("bug method"). Previous work has demonstrated that birds continue to incubate normally during the bug's blood meal (Becker et al. 2006). To determine whether this technique is a viable alternative to conventional trapping and bleeding, we compared baseline corticosterone measurements for the same birds taken via the conventional and bug methods. Specifically, we tested

the prediction that corticosterone levels in blood taken using either method would be statistically indistinguishable.

Materials and methods

Study site

We collected blood samples during the breeding season in 2005 and 2007 at a common tern colony of approximately 490 nesting pairs on the Banter See (53°30′N, 8°06′E), Wilhelmshaven, Germany. Birds at this site have been fitted with passive transponders since 1992, permitting automated identification of marked individuals (Becker and Wendeln 1997, Becker et al. 2001).

Study design

We collected samples via both conventional and bug methods from a total of 28 individuals. In 2005, we selected 14 adult common terns arbitrarily from a subset of marked birds for which we had multi-year data on breeding parameters. Terns initiated nesting between 11–27 May and we sampled blood between 27 May and 16 June (middle to late incubation, median = 15 d after laying of the first egg, range: 11–20 d). In 2007, we selected 14 birds that were either marked with transponders or had a marked mate. These study birds initiated laying between 25 June and 6 July (after peak laying) and we sampled between 14 and 18 July (median = 17 d after laying of first egg, range: 10–21 d).

In both years, we took blood samples from adult common terns using Triatomine bugs *Dipetalogaster maximus* (von Helversen et al. 1986, Becker et al. 2006) between 05:50 and 10:20 CEST. Transponder detections by nest antennas (every 5 s) were checked to ensure that the target bird was incubating. In 2005, we secured dummy eggs containing the bugs to the nest with a 10 cm length of wire and a mesh opening in the dummy egg allowed bugs to draw blood from the brood patch through their proboscises (Fig. 1 a, b; also see Becker et al. 2006). In 2007, to improve the rate of successful bug sampling, we used dummy eggs with a small opening around the circumference of the egg and additional small holes throughout the shell of the egg (Fig. 1 c, d). This design allowed birds to roll the eggs in their nest when settling without obstructing bug feeding.

We checked dummy eggs approximately every 30 min after deployment to determine whether the bugs had fed. Previous work showed that a full blood meal can be obtained within 10 min, although longer periods of time are sometimes necessary (Becker et al. 2006). Nest surveys produced fewer than four small-scale disturbances (<2 min) from deploying dummy eggs to retrieving the sample. These disturbances are unlikely to have affected the stress levels of

Figure 1. Bug method in 2005 (a and b) and 2007 (c and d): a) placing ''dummy'' eggs in common tern *Sterna hirundo* nest with antennae for recording bird activity, two fiberglass eggs (in nest), and one hollow fiberglass egg with bug and with mesh opening to allow bug to feed (in hand)), b) larval instar L3 of *Dipetalogaster maximus* being removed from ''dummy'' egg at completion of a blood meal. c) modified dummy eggs used during 2007 sample collection illustrating the small opening around the egg circumference, and d) removal of *D. maximus* from modified egg in 2007.

study birds because such disturbances are frequent at this colony and birds return to their nests immediately and resume incubation once investigators have moved 2–3 m away. Even so, we included time from initial disturbance to sampling as a covariate in our analysis since longer disturbance times led to more disturbance events.

In both years, we placed nest traps over nests immediately following successful bug sampling. When birds were incubating again, we sprung the trap remotely and collected a blood sample from the brachial vein in a heparinized microcapillary tube within 0-3 min (e.g. Wingfield and Romero 2001). The mean time between bleeding with a bug and trapping was 39 min (median = 15 min; range: 1–160 min, n = 22).

For six birds in 2005, we took samples using a syringe and stored blood with heparin in eppendorf tubes. Due to logistical problems, we were unable to calculate accurate baseline values for these samples and they were excluded from all analyses. Blood samples from three additional individuals for which conventional blood collection times exceeded 3 min were also excluded from analyses, although their corticosterone levels are reported in the results section.

All blood samples were placed on ice and centrifuged within 8 h of sampling. Plasma was removed and stored at -70° C. Corticosterone levels were determined after extraction with diethyl ether in a corticosterone enzyme immunoassay (described in detail in Palme and Möstl 1997).

Statistical analysis

For all analyses, we normalized corticosterone data by log transformation. Summary data from statistical models are presented as back-transformed means with 95% confidence intervals (CI) in the original units.

We used a residual maximum likelihood (REML) model (Patterson and Thompson 1971) in SPSS v. 15 (Norusis 2007) to determine the difference between baseline corticosterone in blood samples collected via the bug and conventional methods. Our analysis included 28 bug samples (14 in each year) and 19 conventional samples (five in 2005 and 14 in 2007). REML can be used in place of ANOVA for unbalanced and repeated measures data (Elston et al. 2001). We controlled for possible effects of year, age, sex, incubation stage (number of days since nest initiation), time from initial disturbance to successful bug bleeding, and time between bug bleeding and trapping on corticosterone concentration. We used year as a covariate but this incorporated the effect of day of season (sampling date relative to colony-wide first egg date) and clutch number (first clutch or replacement clutch) since these three metrics were non-independent. The best model was found by stepwise deletion of non-significant covariate terms (McCullagh and Nelder 1983), with all covariates and biologically meaningful interaction terms initially included in the maximal model. If removal of a term increased the deviance of the model we did not delete the term (Crawley 2005). F-statistics are reported for the effect (sample method) and significant covariates in the best model and for non-significant covariates in the model at the step prior to their removal.

We assessed differences in variance of corticosterone levels between bug and conventional samples using an F-test for equality of variances. This analysis does not include covariates, thus to minimize the potential impact of confounding factors we included only individuals with paired samples collected via both methods (n = 19). We also determined the parametric correlation between corticosterone levels from samples collected via bug and conventional methods for the same individuals.

Results

Mean baseline corticosterone levels were not statistically different when samples were collected via the bug or conventional method (REML: $F_{1,43} = 0.03$, $P = 0.87$, n = 47; mean (95% CI): Bug method = 9.6 (7.9–11.6), Conventional method = 9.3 (7.3–11.9); Table 1). Mean baseline corticosterone levels were higher in 2007 than in 2005, independent of sampling method (2005 season = 6.9 (5.4–8.8) ng/ml, 2007 season = 12.9 (10.6–15.6) ng/ml; Table 1). Overall, males had significantly higher baseline corticosterone levels than females (Males = 10.9 (8.8–13.6) ng/ml, Females = 8.1 (6.6–10.0) ng/ml; Table 1).

Variance in estimated baseline corticosterone levels was not different in samples taken via the bug and conventional methods (F-test: $F_{18} = 1.2$, $P = 0.34$). Corticosterone levels in samples taken by the bug method and by the conventional method were positively correlated ($r^2 = 0.49$, $P < 0.001$, n = 19; Fig. 2).

Untransformed values of baseline corticosterone levels for the 19 individuals with both bug and conventional samples (collected in under 3 min) are presented in Fig. 2. Although we were unable to test whether samples collected after 3 min had significantly higher baseline corticosterone than those collected within this conventional time period,

Table 1. Results of stepwise REML analysis for the effects of sampling method and covariates on corticosterone levels incorporating year as a covariate. Bold text indicates components in the best model. This model had residual df of 43.

Effect or covariate (× denotes interaction)	F-statistic	df	P-value
Time elapsed initial disturbance-bug × Sampling method	0.25	1	>0.05
Time elapsed initial disturbance-bug	< 0.01	1	>0.05
Time elapsed bug-conventional × Sampling method	3.17	2	>0.05
Incubation stage	0.30	1	>0.05
Age	0.45	1	>0.05
Sex	**4.1**	**1**	**0.05**
Year	**16.4**	**1**	**<0.001**
Sampling method	**0.03**	**1**	**>0.05**

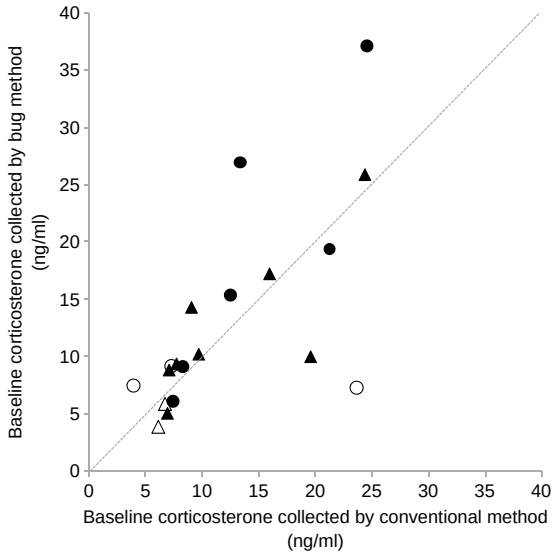

Figure 2. The correspondence between paired measurements of baseline corticosterone concentrations of 19 individual common terns collected via sampling with Triatomine bugs (''bug method'') and via conventional bleeding within 3 min of capture (''conventional method''). Samples taken from males (circles) and females (triangles) in 2005 (open symbols) and 2007 (closed symbols) are indicated. The dotted line indicates where concentrations measured by both methods would be identical.

there were larger deviations in corticosterone concentrations between bug and trap samples when trap samples were drawn between 4–5 min after trapping (n = 1, Bug method: 5.2 ng/ml; Conventional method: 61.8 ng/ml) than when samples were drawn between 3–4 min after trapping (n = 2: Bug method: 6.7 and 4.4 ng/ml, Conventional method: 10.5 and 6.4 ng/ml).

Discussion

Our results indicate that the bug method offers a viable alternative to blood sampling via conventional trapping and bleeding techniques. Mean corticosterone levels in blood samples taken from trapped birds within 3 min of capture were strongly and positively correlated with those of samples taken via the bug method and there were no differences in mean or variance between these two groups.

As corticosterone levels begin to rise within minutes of trapping (Wingfield and Romero 2001, Romero and Reed 2005), we were unable to take blood samples to assess baseline corticosterone using the bug protocol after the birds were trapped. Similarities between hormone concentration in blood samples collected by both methods (Fig. 2) and multiple observations of similar baseline corticosterone in samples collected from an individual using the bug method during the same day (but up to 4 h later; J. M. Arnold and P. H. Becker unpubl. data) imply that the order of sampling did not bias the results.

Although conventional trapping and bleeding has been used for a large range of species at a variety of sites, it has limitations resulting from trap-shyness, sensitivity of birds to disturbance, bird size and required blood-drawing experience that could potentially be overcome by our bug sampling method. Furthermore, repeated sampling of the same individuals is much easier using the bug method than conventional re-trapping. Our study also supports the results of previous assessments of the conventional method that emphasize the importance of obtaining the baseline blood sample within 3 min of trapping (Romero and Romero 2002, Romero and Reed 2005).

Initial validation studies of the bug method for hormone analysis in domestic rabbits *Oryctolagus cuniculus* suggested that digestion or dilution effects of the bug on hormone levels are minimal, particularly when samples are removed from the vector within minutes after the meal is completed (Voigt et al. 2004). Validation studies with herring gulls *Larus argentatus*, in a controlled setting, confirmed this finding (Becker et al. unpubl. data). Studies of domestic rabbits showed that glucorticoid concentrations in bug-drawn blood did not significantly deviate from initial levels, even 24 h after the blood meal (Voigt et al. 2004). Although our results indicate no differences between hormone levels taken via different sampling methods, further validation studies of the bug method, particularly in a field setting, may be warranted.

Baseline corticosterone levels were higher in samples collected in 2007 than in 2005. In 2005, we took samples during the peak incubation period for the colony (mean 37.1 ± 1.5 d after first lay date for the colony) but in 2007 we collected all samples late in the season (mean 73.9 ± 0.4 d after first lay date for the colony) and from second clutches. Consequently, differences in corticosterone between years could be caused either by the day of season at which the sample was taken, clutch number, intrinsic differences in birds breeding at different times in the season or by undetermined year effects (Schoech et al. 1999, Arnold et al. 2004, Buck et al. 2006).

Male common terns had significantly higher baseline corticosterone levels than females, independent of year (Table 1). In red-footed boobies *Sula sula*, higher levels of corticosterone were found in males than in females during chick-rearing (Lormée et al. 2003), though many authors have reported no sex differences for seabirds (Angelier et al. 2006, 2007, Chastel et al. 2006), including common terns (Heidinger et al. 2006). Sex-specific foraging demands of common terns during incubation (Becker and Ludwigs 2004) may account for the slightly higher baseline levels we observed among males.

Our results suggest that the bug method offers a viable alternative to sampling via the conventional technique in situations where birds are difficult to trap, or trapping introduces undue stress to the study organism. We found no evidence that terns were disturbed by feeding bugs, whereas birds that we trapped often spent several hours off their nest after being handled and bled by the conventional method. The bug method was used in 2007 with a success rate of 89.5% (C. Bauch unpublished data) and is cost effective, minimally-invasive and requires little training (Voigt et al. 2005, 2006). In species where conventional blood-drawing is difficult, bugs have also been applied to trapped individuals, in lieu of needles, to avoid hematomas (Voigt et al. 2004).

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References

Angelier, F., Shaffer, S. A., Weimerskirch, H. and Chastel, O. 2006. Effect of age, breeding experience and senescence on corticosterone and prolactin levels in a long-lived seabird: the wandering albatross. – Gen. Comp. Endocrinol. 149: 1–9.

Angelier, F., Shaffer, S. A., Weimerskirch, H., Trouvé, C. and Chastel, O. 2007. Corticosterone and foraging behavior in a pelagic seabird. – Physiol. Biochem. Zool. 80: 283–292.

Arnold, J. M., Hatch, J. J. and Nisbet, I. C. T. 2004. Seasonal declines in reproductive success for the common tern: timing or parental quality? – J. Avian Biol. 35: 33–45.

Arnold, J. M., Nisbet, I. C. T. and Hatch, J. J. 1998. Are common terns really indeterminate layers? Responses to experimental egg removal. – Colon. Waterbird. 21: 81–86.

Becker, P. H. and Ludwigs, J.-D. 2004. *Sterna hirundo* common tern. – In: Parkin, D. (ed.). BWP update 6: 91–137.

Becker, P. H. and Wendeln, H. 1997. A new application for transponders in population ecology of the common tern. – Condor 99: 534–538.

Becker, P. H., Voigt, C. C., Arnold, J. M. and Nagel, R. 2006. A non-invasive technique to bleed incubating birds without trapping: a blood-sucking bug in a hollow egg. – J. Ornithol. 47: 115–118.

Becker, P. H., Wendeln, H. and González-Solís, J. 2001. Population dynamics, recruitment, individual quality and reproductive strategies in common terns *Sterna hirundo* marked with transponders. – Ardea 89: 241–252.

Buck, C. L., O'Reilly, K. M. and Kildaw, S. D. 2006. Interannual variability of Black-legged Kittiwake productivity is reflected in baseline plasma corticosterone. – Gen. Comp. Endocrinol. 150: 430–436.

Chastel, O., Lacroix, A., Weimerskirch, H. and Gabrielsen, G. W. 2006. Modulation of prolactin but not corticosterone responses to stress in relation to parental effort in a long-lived bird. – Horm. Behav. 47: 459–466.

Crawley, M. J. 2005. Statistics: an introduction using R. – John Wiley and Sons, Hoboken.

Elston, D. A., Horgan, G. W. and Hunter, E. A. 2001. Introduction to REML for scientists. – BioSS, Edinburgh.

Heidinger, B. J., Nisbet, I. C. T. and Ketterson, E. D. 2006. Older parents are less responsive to a stressor in a long-lived seabird: a mechanism for increased reproductive performance with age? – Proc. R. Soc. B 273: 2227–2231.

Hofer, H. and East, M. L. 1998. Biological conservation and stress. – In: Møller, A.P., Milinski, M. and Slater, P. J. B. (eds). Advances in the study of behavior. Vol. 27. Stress and behavior. – Academic Press, San Diego, pp. 405–524.

Lõhmus, M., Sandberg, R., Holberton, R. L. and Moore, F. R. 2003. Corticosterone levels in relation to migratory readiness in red-eyed vireos (*Vireo olivaceus*). – Behav. Ecol. Sociobiol. 54: 233–239.

Lormée, H., Jouventin, P., Trouve, C. and Chastel, O. 2003. Sex-specific patterns in baseline corticosterone and body condition changes in breeding red-footed boobies *Sula sula*. – Ibis 145: 212–219.

McCullagh, P. and Nelder, J. A. 1983. Generalized linear models. – Chapman & Hall, London.

Millspaugh, J. J. and Washburn, B. E. 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. – Gen. Comp. Endocrinol. 138: 189–199.

Nisbet, I. C. T. 1981. Behavior of common and roseate terns after trapping. – Colon. Waterbird. 4: 44–46.

Norusis, M. 2007. SPSS 15.0 Advanced statistical procedures companion. – Prentice Hall, Upper Saddle River.

Palme, R. 2005. Measuring fecal steroids: guidelines for practical application. – Ann. NY Acad. Sci. 1046: 75–80.

Palme, R. and Möstl, E. 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. – Z. Saugetierkd. – Int. J. Mamm. Biol. 62 (Suppl. II): 192–197.

Patterson, H. D. and Thompson, R. 1971. Recovery of interblock information when block sizes are unequal. – Biometrika 58: 545–554.

Romero, M. L. and Reed, M. J. 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? – Comp. Biochem. Physiol. A 140: 73–79.

Romero, M. L. and Romero, R. C. 2002. Corticosterone responses in wild birds: the importance of rapid sampling. – Condor 104: 129–135.

Schoech, S. J., Ketterson, E. D. and Nolan, V. 1999. Exogenous testosterone and the adrenocortical response in dark-eyed juncos. – Auk 116: 64–72.

Touma, C. and Palme, R. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. – Ann. NY Acad. Sci. 1046: 54–74.

Voigt, C. C., Fassbender, M., Dehnhard, M., Wibbelt, G., Jewgenow, K., Hofer, H. and Schaub, G.A. 2004. Validation of a minimally invasive blood-sampling technique for the analysis of hormones in domestic rabbits, *Oryctolagus cuniculus* (Lagomorpha). – Gen. Comp. Endocrinol. 135: 100–107.

Voigt, C. C., Michener, R., Wibbelt, G., Kunz, T. H. and von Helversen, O. 2005. Blood-sucking bugs as a gentle method for blood-collection in water budget studies using doubly labelled water. – Comp. Biochem. Physiol. A 142: 318–324.

Voigt, C. C., Peschel, U., Wibbelt, G. and Frölich, K. 2006. An alternative, less invasive blood sample collection technique for serologic studies utilizing Triatomine bugs (Heteroptera; Insecta). – J. Wildlife Dis. 42: 466–469.

von Helversen, O., Volleth, M. and Nunez, J. 1986. A new method for obtaining blood from a small mammal without injuring the animal: use of Triatomid bugs. – Experientia 42: 809–810.

Walker, B. G, Boersma, P. and Wingfield, J. C. 2005. Physiological and behavioural differences in magellanic penguin chicks in undisturbed and tourist-visited locations of a colony. – Conserv. Biol. 19: 1571–1577.

Wingfield, J. C. and Romero, L. M. 2001. Adrenocortical responses to stress and their modulation in free-living vertebrates. – In: McEwen, B. S. and Goodman, H. M. (eds). Handbook of physiology; Section 7: the endocrine system; Vol. IV: coping with the environment: neural and endocrine mechanisms. – Oxford Univ. Press, New York, pp. 211–234.